Lentiviral Production
(Welm et al. (2008), *Cell Stem Cell* **2**: 90-102)

Media

HEK Medium:

DME-H21	(Gibco cat # 11995, 450 ml per 500 ml)
10% FBS	50 ml per 500 ml

MEC Growth Medium (to resuspend concentrated virus):

DMEM/F-12	(Gibco cat # 11330, 442 ml per 500 ml)
10% FBS	50 ml per 500 ml
100 U/ml Penicillin G 100 μg/ml Streptomycin	5 ml 100x Pen/Strep per 500 ml
5 μg/ml Insulin	2.5 ml of 1 mg/ml per 500 ml
50 μg/ml Gentamicin	500 μl of 50 mg/ml per 500 ml
1 μg/ml Hydrocortisone	50 μl of 10 mg/ml (in 100% EtOH) per 500 ml
10 ng/ml mouse EGF	(Roche cat # 855-731, 50 μl of 100 μg/ml per 500 ml)

All media should be sterilized through a 0.22 μm filter and can be stored up to one month at 4 °C!

Solutions

1xHBS:

21 mM HEPES	2.5 g per 500 ml
137 mM NaCl	4.0 g per 500 ml
5.56 mM Dextrose	0.5 g per 500 ml
50 mM KCl	1.85 g per 500 ml
1.4 mM $Na_2HPO_4(7H_2O)$	10 ml of *stock solution

* stock solution (70 mM): 0.94 g of $Na_2HPO_4(7H_2O)$ in 50 ml HPLC purified H_2O

1) Add HPLC purified H_2O up to ca. 450 ml and dissolve chemicals
2) Adjust pH with NaOH to **exactly** 7.1
3) Add HPLC purified H_2O up to 500 ml
4) Sterilize through a 0.22 μm filter
5) Make 40 ml aliquots in sterile 50 ml screw cap tubes and store at -20°C. Keep one working tube at 4 °C.

2.5 M $CaCl_2$:
Dissolve either 27.75 g of anhydrous $CaCl_2$ or 36.75 g of $CaCl_2(2H_2O)$ in 100 ml of HPLC purified H_2O and sterilize through a 0.22 μm filter. Make 10 ml aliquots in sterile 15 ml screw cap tubes and store at -20°C. Keep one working tube at 4 °C.

Calcium phosphate-mediated transfection of HEK-293T cells

Day 1

1) In the late afternoon (± 4 pm) of the day before transfection, split an almost confluent 10-cm dish of HEK-293T cells 1:5 in HEK medium. This should give you about $5x10^6$ cells per dish. Gently triturate the trypsin/cell mixture to avoid cell clumps, as these reduce your transfection efficiency. Make sure your plates are level for an even cell distribution.

2) Pre-warm an appropriate amount of HEK medium overnight.

Day 2

3) The plates should be 50-60% confluent early next morning. Aspirate the culture supernatant and carefully add 10 ml of fresh HEK medium to each plate by holding your pipette against the side of the dish. This can be a time-consuming procedure, especially if you have a lot of plates.

4) Set up DNA mixtures for your transfections; you can do this at your bench, as long as you use sterile pipette tips and tubes. The calcium phosphate procedure requires 30μg of total DNA per 10-cm dish (see www.flemingtonlab.com for experimental design considerations). However, I routinely use only 24μg of total DNA per 10-cm dish: 12 μg of transfer vector (ie HIV-ZsGreen/pEIZ), 4 μg of the envelope vector pVSV-G (Clontech) and 4 μg each of the packaging vectors pMDLg/pRRE and RSV-REV (Dull *et al.*, 1998). If your transfer vector contains a large (≥1.5 kb) insert, you can partially compensate for this by increasing the amount of transfer vector up to 18 μg.

I like to work with 4x10-cm dishes per lentiviral construct, as this yields the exact amount of viral supernatant that will fit in one of our ultracentrifuge tubes. **Therefore, the following amounts are for transfection of 4x10-cm dishes!**

5) For each lentiviral construct, pipette in a tissue culture hood 2 ml of 1xHBS in a sterile 15 ml screw cap tube.

6) Add DNA mixture for 4x10-cm dishes and briefly vortex to mix.

7) Add 120 μl of 2.5 M $CaCl_2$, immediately vortex and let sit for 20 min.

8) To ensure proper buffering of the culture medium, remove only one stack of 4x10-cm dishes from the 5% CO_2 incubator at a time. Add one quarter of the transfection mixture to each plate in a drop wise fashion and mix by rocking back and forth (don't swirl!). Put the stack of 4x10-cm dishes immediately back in a 5% CO_2 incubator. Repeat step 8 as many times as necessary.

9) Again, pre-warm an appropriate amount of HEK medium overnight. For convenience I like to prepare 40 ml aliquots per lentiviral construct, to prevent cross-contamination of my viral preps.

Assume from here on that anything coming into contact with either the culture supernatant or the HEK cells is contaminated with lentivirus! **Thus, always wear a lab-coat and double glove for your own protection. Have spray bottles with a fresh (less than one-week-old) 10% bleach solution or 70% EtOH handy, to clean up any spills. Briefly rinse used Pasteur pipettes with 10% bleach, before discarding them in the sharps container. Soak regular plastic pipettes in a waste beaker filled with 10% bleach for at least 30 min before discarding them in the Biohazard waste container. Read and understand your lab's BUA protocol!**

Collection of viral supernatant

Day 3

1) The next morning, carefully replace the culture supernatant of each dish with 10 ml pre-warmed HEK medium. This step removes excess calciumphosphate precipitate, which you otherwise would pellet during ultracentrifugation. This precipitate interferes with the resuspension of the viral particles and negatively affects primary MECs.

2) Later in the afternoon, prepare more 40 ml aliquots of HEK medium and pre-warm overnight.

Day 4

3) The next morning, carefully collect the 24 h viral supernatant of each plate and replace with 10 ml pre-warmed HEK medium.

4) Combine all viral supernatants of the same lentiviral construct in a 50 ml screw cap tube and pre-clear by spinning at 12,000 x *g* for 5 min.

5) Afterwards, pass the viral supernatant through a 0.45 μm syringe PES filter (Nalgene cat # 194-2545) and store at 4 °C (do NOT freeze!) until further processing.

Day 5

6) Carefully collect the viral supernatant and add 3 ml of 10% bleach to each plate. Let soak for at least 30 min before discarding plates.

7) Combine all viral supernatants of the same lentiviral construct in a 50 ml screw cap tube and pre-clear by spinning at 12,000 x *g* for 5 min. This step is especially important for collection after 48 h: there is quite a bit of cell debris and you will notice a fairly large pellet.

8) Pass the viral supernatant through a 0.45 μm syringe PES filter and store at 4 °C until further processing. Despite the pre-clearing step, you will likely need two or even three filters per 40 ml of 48 h viral supernatant.

Concentration of viral supernatant

Our lab has a Beckman L8-70M Ultracentrifuge with a SW-27 rotor. The following protocol has been optimized for this particular centrifuge. If you have never used this ultracentrifuge before, you should practice attaching the buckets to the rotor as well as putting the rotor onto the spindle of the ultracentrifuge. These are tricky steps to do for the first time, especially when the buckets are filled to the rim with lentiviral supernatant.

Day 5

1) Sterilize the appropriate number of inner, thin wall Ultra-Clear™ centrifuge tubes (Beckman cat # 344058) by inverted (place hand with clean glove on top!) rinsing with 100% EtOH. Place tubes up side down on a paper towel inside the tissue culture hood. Make sure that they completely dry by moving them around on the paper towel. Mark the tubes to prevent confusion and/or viral cross contamination.

2) Clean the swinging buckets with 70% EtOH and place them inside the tissue culture hood. Make sure that the interior is completely dry and that the rubber O-ring is intact.

3) Clean a scale with 70% EtOH and place inside tissue culture hood. Clean a Styrofoam block that can hold 50 ml screw cap tubes with 70% EtOH, and place on top of the scale.

4) Place a swinging bucket without cap on the Styrofoam block and insert the inner centrifuge tube. Make sure that there is no residual EtOH, as this will inactivate your virus. Add 37 ml of the appropriate 0.45 μm filtered viral supernatant and loosely screw on cap. Repeat step 4 as many times as necessary.

To prevent collapse of the inner tube, your volume should never be less than 37 ml!

5) To balance, turn of the blower and compare the weight of bucket # 1 to # 4, # 2 to # 5 and # 3 to # 6. They usually are within 200 μg (ie 200 μl) of each other; I aim for identical weight, but a difference of 10 μg (ie 10 μl) is acceptable. Screw the caps on tight and walk the buckets over to the ultracentrifuge.

If you have only a few samples to concentrate, you should still balance the remaining empty buckets and attach them to the rotor, so that each bucket has experienced the same amount of g forces.

6) Turn the ultracentrifuge on and inspect the interior; remove oil residue with 70% EtOH and a paper towel. Attach each bucket to its correct location on the rotor and carefully place the assemblage on the spindle. Program the ultracentrifuge:

- Press Speed; enter 25000 rpm (= 100,000 x *g*); Press Enter/Display
- Press Temp; enter 4 °C; Press Enter/Display
- Press Time; enter 1 h 45 min; Press Enter/Display
- Press Start

The centrifuge will initially spin up to 3000 rpm. If the rotor is not properly balanced, the centrifuge will shut down and give an error message. If the rotor is properly balanced, the centrifuge will start spinning faster as soon as the vacuum drops below 700 μm, which should not take longer than 5 min. At the end of the run, the centrifuge will beep and you have to release the vacuum by pushing the Vacuum button. Make sure you enter the number of rotations in the maintenance logbook, before you start the next run. You also have to remove the condensation on the interior of the centrifuge with some paper towel and 70% EtOH, as this may cause harm to the vacuum trap.

7) Have a beaker with 10% bleach, sterile forceps, 10 ml of MEC growth medium, sterile glass pipettes and some pieces of parafilm ready in your tissue culture hood. Attach a glass pipette to your aspirator and open a bucket inside the tissue culture hood. Carefully pull out the inner centrifuge tube with the forceps and pore the supernatant into the 10% bleach beaker in one continuous motion. Keep the tube up side down and aspirate residual HEK medium from the tube wall with the glass pipette. The viral particles form a clear to light brown pellet on the bottom of the tube. Add desired amount of MEC growth medium (200 μl = 185-fold and 100 μl = 370-fold concentration) to the bottom of the tube and seal with a piece of parafilm. Process all buckets before you resuspend the viral pellets.

8) Set your P-200 pipetteman to either 150 or 75 μl, depending on the resuspension volume. Gently triturate the virus/MEC growth medium mixture to avoid air bubbles, as these reduce your viral yield. The pellet should gradually break up and dissolve. Put tubes at 4 °C for 2 hours to promote resuspension of the virus.

9) Briefly triturate again and combine pellets of the same lentiviral construct to reduce titering efforts. Mix by gently vortexing and aliquot the concentrated virus: I usually make 50 μl aliquots to avoid repeated freeze/thaw cycles, and designate one 15 μl aliquot for titration. Store the aliquots at -80 °C; the concentrated virus should be stable for at least 6 months.

Determining viral titer

Day 4

1) In the afternoon of the day before titration, split $2x10^5$ HEK-293T cells per well of a six-well plate in HEK medium. You will need two wells per lentiviral construct, four wells for counting and at least one well as a negative control for FACS. If possible generate one appropriately diluted HEK-293T cell mixture, so that all wells will have the exact same number of cells.

Day 5

2) The wells should be 10-20% confluent the next day. Count the number of cells in four wells;

- briefly rinse each well with 0.5 ml of trypsin
- add 200 μl of trypsin and incubate at 37 °C for 5 min
- add 300 μl of HEK medium and collect the cells by trituration
- count five fields per well and calculate the average cell number (make sure you correct for the 0.5 ml final cell volume!)

3) Retrieve the 15 μl viral aliquots from the -80 °C and make 1:10 dilutions in HEK medium.

4) Carefully replace the culture supernatant of the remaining wells with 2 ml pre-warmed HEK medium.

5) Mark two wells for each lentiviral construct; add 2 μl of diluted viral concentrate to one well (ie 1:10,000 dilution) and 20 μl to the other well (ie 1:1,000 dilution). Mix by gently rocking back and forth (don't swirl!). Place the six-well plates back into the incubator and leave the viral supernatant on the cells for at least 60 hours to allow for expression of the fluorescent marker. I typically titer on Friday afternoon and analyze my cells by FACS on Monday morning.

Day 8

6) After 60-72 hours, visually inspect your cells on a fluorescent tissue culture scope to see whether the infection worked. Determine the % of fluorescent cells by FACS and calculate the titer in transducing units per milliliter (TU/ml) by using

the following formula; [cell number at time of virus addition x (% fluorescent cells/100)] x 1000 / μl of viral concentrate. For accurate titering, only use infections that result in fewer than 15% fluorescent cells, as these represent one viral integration per cell. We routinely get 10^8 TU/ml for empty and 10^7 TU/ml for viruses with an insert. Titers of ≤ 10^6 TU/ml are not suitable for infection of primary MECs.

Tips and Suggestions

- The un-concentrated viral supernatant is stable at 4 °C for several weeks, while freezing results in a significant reduction in titer.

- ± 50% of the virus is lost during concentration and storage at -80 °C.

- The viral titer after 24 h is on average 5 x higher than after 48 h. Thus, only concentrate the 24 h viral supernatant if you have difficulty getting high viral titers.

- Different cell lines will give different viral titers. We only titer on HEK-293T cells, as they give reproducible viral titers over a wide range of confluency. In addition, our in suspension infection method of primary MECs was optimized with virus titered on HEK-293T cells.

- Viral titers may also vary depending on the fluorescent marker of the transfer vector. For instance, ZsGreen of HIV-ZsGreen/pEIZ is easier to detect by FACS than H2BmRFP of HIV-H2BmRFP/pEIR, which may explain the log difference in viral titer for these two transfer vectors.

- Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. We found that this compound did NOT increase the transduction efficiency of primary MECs during the overnight in suspension infection, and we therefore do not include it in our titering protocol.